Journal of Colloid and Interface Science 430 (2014) 40–46

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Interaction between lysozyme and humic acid in layer-by-layer assemblies: Effects of pH and ionic strength

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ARTICLE INFO

Article history:
Received 11 April 2014
Accepted 20 May 2014
Available online 2 June 2014

Keywords:
Humic acid
Protein
Lysozyme
Protein encapsulation
Layer-by-layer adsorption
2-D assembly
Reflectometry
3-D assembly
Particle charge detector

ABSTRACT

The interaction between protein and soluble organic matter is studied through layer-by-layer assembly of lysozyme (LSZ) and purified Aldrich humic acid (PAHA) at a solid surface (2-D) and in solution (3-D). By bringing a silica surface in alternating contact with solutions of LSZ and PAHA a layer-by-layer LSZ–PAHA assembly is formed. At pH 5 the negative charge density of PAHA is about 3 times that of the positive LSZ; the layers of LSZ and PAHA are stable and the adsorbed amounts decrease with increasing ionic strength. The mass ratios PAHA/LSZ in the layers depend on the ionic strength; K^+ incorporation is relatively large (~25%) when PAHA is the outer layer of the assembly. At pH 6 and 8, and moderate ionic strength (0–100 mmol L^{-1} KCl) the assembly is accompanied by partial solubilization of positive LSZ by the much more negative PAHA followed by desorption of the complex. The solubilization increases with increasing pH, and decreases with increasing KCl concentration. At 400 mmol L^{-1} KCl the electrostatic interactions are so well screened that the assembly is no longer accompanied by layer erosion. Assembly of PAHA and LSZ in solution is also investigated at pH 5 and 5 mmol L^{-1} KCl. The PAHA/LSZ mass ratio at the iso-electric point of the assembly depends on the order of the addition. When LSZ is added to the negative assembly K^+ is incorporated in the complex, but when PAHA is added to the positive assembly PAHA and LSZ neutralize each other.

1. Introduction

Enzymes and other proteins in the natural environment derived from plants, animals and, foremost, microorganisms, play an important role in the biogeochemical cycle of C, N, P, S and metals. However, due to human activity also toxic and infectious proteins, including insecticidal Cry proteins from genetically modified Bt crops and prion proteins derived from animal excreta or carcasses causing scrapie in sheep and Bovine Spongiform Encephalopathy ('mad cow disease') in bovine livestock can spread in the environment [1–3]. In natural environments most proteins in solution are readily degradable, e.g., by proteolysis, but they may be protected against degradation by adsorption to and encapsulation in other organic substances [4–7]. However, adsorption and encapsulation may also reduce the activity of enzymes by screening the active center from being accessed by the substrate [6,8]. In the studies on protein encapsulation in natural organic matter soluble humic substances (HSs) were used as model organics [6–11]. Soluble humic substances are composed of humic and fulvic acids (HAs and FAs). HAs are chemically heterogeneous soft particles that easily aggregate, especially at lower pH values and that have sizes comparable to that of small globular proteins [12–14]. FAs have a chemistry that is similar to HAs, but they have a higher degree of oxidation, a smaller molar mass, and a higher solubility. At natural pH values HAs and FAs are negatively charged due to deprotonation of carboxylic and phenolic functional groups [15–17].

Detailed studies on protein–HS interaction in solution are limited. The binding of small peptides to HS has been investigated by nuclear magnetic resonance spectroscopy [7,18]. In an extensive study on protein–HA complexation, based on proton titrations, dynamic light scattering and isothermal titration calorimetry, as a function of protein/HA mass ratio, pH and ionic strength, Tan et al. [9,10] concluded that encapsulation of the proteins was driven by electrostatic attraction between positively charged parts of the protein surface and negatively charged HA, and by hydrophobic attraction. The encapsulation was at a maximum when the protein–HA complex was at its iso-electric point (IEP) [9]. A study on complexation between lysozyme (LSZ) and HA

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http://dx.doi.org/10.1016/j.jcis.2014.05.029
0021-9797/

revealed that the lysozyme activity reduced upon complexation, depending on the LSZ/HA mass ratio in the complex, the pH, and the ionic strength [8].

HA and FA films coat many surfaces in soils, particularly those of positively charged metal (hydr)oxides [19–21] and proteins may adsorb at negative, positive and neutral natural surfaces [22,23]; therefore, also encapsulation of proteins in HAs and FAs films at surfaces is relevant. In vitro, layer-by-layer assemblies may be formed when alternately added oppositely charged polyelectrolytes assemble on top of each other [24]. For each layer, the last-added polyelectrolyte binds super-equivalently such that the charge sign of the assembly reverses and, hence, facilitates binding of the oppositely charged polyelectrolyte to be supplied in the next addition. The layer-by-layer adsorption technique has been used to assess the activity of enzymes encapsulated in films of polyelectrolytes [25], to investigate the association of HS and positively charged polyelectrolytes [26] and the association of HS and proteins at solid–aqueous solution interfaces [11,27,28]. Tomaszewski et al. [11] studied the encapsulation of lysozyme, ribonuclease-A, and trypsin by various terrestrial and mixed terrestrial–aquatic HAs and FAs over a range of pH values, and concluded that electrostatic attraction between positively charged regions of the proteins and the negatively charged HS is a major driving force for encapsulation. However, encapsulation of lysozyme at pH 8 and of ribonuclease-A at pH 5 and 6 involved partial disassembly of HA supramolecular association. Subsequently, Sander et al. [27] and Tomaszewski et al. [28] studied Cry1-Ab protein binding to HSs bound to silica covered with poly-lysine and found that also the polarity of the HSs was an important factor. In general, both the results of Tan et al. [9,10] on protein–HS encapsulation in solution and those of Tomaszewski et al. [11,28] and Sander et al. [27] on protein encapsulation in surface layers indicate that next to electrostatic interactions also other favorable interactions, such as hydrophobic attraction, are important since they may increase the overall attraction [10,28] or overcome local protein–HS electrostatic repulsion [11].

The strength of electrostatic attraction between oppositely charged polyelectrolytes is in the first place determined by their charge densities, which for weak polyelectrolytes, like proteins and HS, are determined by the pH. Secondly, the ionic strength is of generic importance, as it screens the electrostatic interactions. For protein–HS interaction in solution it has been shown that the ionic strength not only screens the electrostatic interactions but that also specific effects may occur due to ion incorporation in the protein–HS complexes [9]. The ease of formation of layer-by-layer assemblies of HS and proteins is therefore determined by both the pH and ionic strength. Partial disassembly of components from layered weak polyelectrolyte protein systems has been described before [29–35] and the ionic strength is of known importance for the 'dissolution' of layer-by-layer assemblies [36]. Also the partial disassembly of HA supramolecular associations as described in Tomaszewski et al. [11] is likely dependent on pH and ionic strength, but a systematic investigation is lacking. Therefore, in the present study attention will be paid to the effects of pH and ionic strength on protein–HS layer-by-layer assembly and partial disassembly. For our study LSZ and purified Aldrich humic acid (PAHA) were chosen as model components because their interaction in solution is well studied [8–10]. In the important pH window 4–9 LSZ is positively charged and PAHA negatively charged and layer-by-layer assemblies of these components can be formed on a silica surface. The main emphasis is on layer-by-layer adsorption on the silica surface (2-D assemblies), but some attention will be given to LSZ–PAHA assemblies built-up in solution by sequential addition of PAHA and LSZ solutions to a PAHA/LSZ complex (3-D assemblies). Measurements on 2-D surface assemblies were carried out *in situ* using stagnation-point reflectometry, and 3-D solution assemblies were made by titration and studied using a particle charge detector.

2. Experimental section

2.1. Materials

2.1.1. Water and chemicals

Water was de-ionized twice and filtered through an activated carbon column and a micro-filter (EASYpure UV); it had a resistance greater than 18.3 MΩ cm. The inorganic chemicals used were of analytical grade quality (obtained from Merck or Sigma–Aldrich).

2.1.2. Humic acid (PAHA)

Aldrich humic acid (Aldrich H1, 675-2) was purified following Aiken et al. [37] and Thurman et al. [38]. To 1 L aqueous solution containing 5 mL of concentrated HF and 5 mL of concentrated HCl 10 g of humic acid was added. The solution was stirred for 8 h and filtered over a Whatman Cellulose filter, grade 2, to remove silica and other soluble minerals. The humic substance residue was washed several times with 1 mol L^{-1} HCl to remove a possible fulvic acid fraction. After neutralization the residue was dissolved in a NaOH solution of pH 9 for 24 h and filtered to remove possible humin. The filtrate was brought to pH 2 with 1 M HCl, stirred for 24 h, and centrifuged. The humic acid precipitate was dialyzed against slowly flowing purified water until the resistance values before and after dialysis were equal (about 3–4 weeks). After freeze-drying the purified Aldrich HA (PAHA) is stored in a desiccator. Studies of various humic acids including PAHA have shown that the physicochemical behavior of PAHA is similar to that of other humic acids [39–41]. The PAHA molar mass as determined by viscometry and gel permeation chromatography is around 20 kDa. The elemental analysis on an ash free basis of PAHA is: C, 55.8%; O, 38.9 %; H, 4.6%; N, 0.6% (wt) [21]. Charge density data of PAHA [9] at the relevant pH and KCl concentrations are collected in Table S1. Stock solutions of PAHA (2 g L^{-1}) were made in measuring flasks by dissolving PAHA at pH 10. The high pH ensures that the HA is well dissolved [42].

2.1.3. Lysozyme (LSZ)

Hen egg-white lysozyme (L-6876; Molar mass 14.6 kDa) was purchased from Sigma and used without further purification. A space filling model of hen egg-white LSZ has been presented by Horsley et al. [43]; LSZ has a nearly spherical shape and a good structural stability [44]. The IEP of LSZ is around 10.5. Relevant charge density data of LSZ [9] are collected in Table S1. The LSZ was dissolved in purified water to a concentration of 5 g L^{-1}. Other LSZ solutions were made from this stock solution. The LSZ stock solution was stored in a refrigerator at 5 °C to prevent degradation. LSZ is colloidally stable in solutions ranging from moderately acidic to basic; even at the IEP no serious flocculation occurs.

2.2. Methods

2.2.1. Reflectometry

Layer-by-layer assembly of LSZ and PAHA on a flat silica surface has been monitored by means of reflectometry, in a stagnation point (impinging jet) flow cell, as described by Dijt et al. [45–47]. The impinging jet allows accurate control over the transport of molecules toward the substrate and effectively shields the observed part of the surface from molecules or particles other than those carried by the jet. With reflectometry a polarized laser beam is reflected from a surface at a fixed angle of incidence. After reflection the perpendicular (I_s) and the parallel intensities (I_p) of the